Survival of *Vibrio cholerae* and *Escherichia coli* in Estuarine Waters and Sediments

MARY A. HOOD* AND GREGORY E. NESS

Department of Biology, The University of West Florida, Pensacola, Florida 32504

Received 12 August 1981/Accepted 30 November 1981

In in vitro estuarine water and sediment chambers, the survival of *Vibrio cholerae* and *Escherichia coli* was determined by plate counting and direct counting techniques. *V. cholerae* strains included environmental, clinical, and serotype O1 and non-O1 isolates, whereas *E. coli* strains included ATCC 25922 and a freshly cultured human isolate. Recovery of *V. cholerae* varied significantly with incubation temperature. Growth and extended periods of survival occurred in sterile sediments, sterile waters, and nonsterile waters, but not in nonsterile sediments. In contrast to *V. cholerae*, viable cells of *E. coli* decreased rapidly in both sterile and nonsterile estuarine waters. Direct counts revealed that *E. coli* cells were intact in the estuarine water, but attempts to resuscitate them were unsuccessful. The data suggest that *V. cholerae* survives better in estuarine waters than *E. coli*. The results may explain the recent observations that *V. cholerae* levels do not correlate well with fecal coliform concentrations in estuarine waters. Furthermore, the results add increasing evidence to support the theory that *V. cholerae* is an autochthonous bacterium in estuaries.

The common occurrence of *Vibrio cholerae* in the estuary (7, 8, 13, 14, 18) has led to the conclusion that the organism is an indigenous resident of the estuary (8, 14). Furthermore, there is increasing evidence suggesting that the presence of *V. cholerae* does not correlate well with levels of fecal coliforms (8, 13, 14). The possibility that fecal coliforms may not indicate the presence of *V. cholerae* in estuarine waters is especially significant from a public health standpoint, since fecal coliforms have historically been used as indicators of water and shellfish quality. With the recent outbreaks of cholera and gastrointestinal disorders caused by *V. cholerae* (2, 4–6, 20) and traced to shellfish, the question becomes even more relevant.

There have been many studies on the survival of the coliform *Escherichia coli* in seawater (10, 19) and a number of early works on the survival of *V. cholerae* in seawater as reviewed by Pollitzer (16). Survival of coliforms in marine waters was concluded to be a function of salt concentration, predation, competition by native microflora, heavy metals, and nutrients (3, 11, 15). *V. cholerae* survival in seawater was found to be related to temperature, pH, salt concentration, the amount of organic material, and the degree of bacterial contamination (16). Although these studies have reported the survival rates of *E. coli* and *V. cholerae*, there is no comparative information on the survival of these two organisms in estuarine environments. Thus, it was the purpose of this study to examine the survival of strains of *V. cholerae* in estuarine waters and sediments and to compare their survival to that of *Escherichia coli*.

MATERIALS AND METHODS

**Survival chambers.** Water and sediment were collected from Apalachicola Bay, Fla., with a Ponor grab (Wildlife Supply Co., Saginaw, Mich.) and sterile water bottles. The sediment samples were placed on ice, returned to the laboratory, and kept at 4°C until the survival chambers were established (within 5 days after collection). Water samples were stored at ambient temperature and filtered through a Whatman no. 1 filter to remove large detrital particles. Salinities of the waters ranged from 6 to 20%. Into sterile glass containers (200 ml) designated the survival chambers, 100 ml of the collected estuarine water was added. Sediment chambers were prepared with 50 g of sediment and 50 ml of estuarine water. The sterile water and sediment chambers were autoclaved at standard conditions. All chambers were allowed to equilibrate at 20°C for 48 h before inoculation, and two or three replica chambers were prepared for each experiment.

**Culture preparation.** Environmental *V. cholerae* strains were isolated from oysters collected from Apalachicola Bay by the methods of Hood et al. (13) and Kaper et al. (14) and were maintained on Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.)–1% NaCl agar. *V. cholerae* strains included an environmental O1 Inaba, WF/1 (13), an estuarine water-adapted O1 strain, five environmental non-O1 strains, WF/23, WF/54, WF/103, WF/108, and WF/111, and a clinical O1 strain (6). Non-O1 *V. cholerae* strains were typed by Ron Siebeling, Louisiana State University, Baton Rouge, as TT (WF/23), Unk (WF/54, WF/108,
and WF/103), and BB (WF/111). The estuary-adapted isolate was strain WF/1 placed in estuarine waters. The organism was recovered from the water after 6 weeks and revitalize on Trypticase soy broth (BBL). The E. coli strains included ATCC 25922 and a freshly isolated strain from human feces. E. coli strains were biochemically confirmed with the API 20E system (Analytab Products, Plainview, N.Y.).

V. cholerae and E. coli isolates were grown in Trypticase soy broth–1% NaCl for 24 h at 35°C. Cultures were centrifuged at 2,000 rpm for 20 min and washed in phosphate-buffered saline. The procedure was repeated twice, and the cells were inoculated into the survival chambers. Incubation temperatures, initial inoculum size, and time and location of water and sediment collection were varied.

Recovery of viable cells. At selected time intervals, samples were taken from the chambers and diluted in phosphate-buffered saline blanks. Enumeration was performed by the standard plate count method on brain heart infusion agar (Difco Laboratories, Detroit, Mich.), Trypticase soy–1% NaCl agar, and thiosulfate citrate bile salt sucrose agar (Difco) for V. cholerae. For E. coli recovery, eosin methylene blue (EMB) agar (Difco) and brain heart infusion agar were used. Enumeration of V. cholerae and E. coli colonies was performed by comparing them with control colonies. O1 strains were confirmed by using polyvalent antiserum (Difco), whereas non-O1 serotype and E. coli colonies were confirmed by using the API 20E System. In addition, the most probable number for fecal coliforms was determined by using the standard method with lauryl tryptose broth (Difco) and incubation at 35°C for 48 h (1). Positive tubes were transferred to EC broth (Difco) and incubated at 44.5°C for 48 h. Positive tubes were then streaked onto EMB agar. Attempts were made to resuscitate the E. coli cells into lactose broth (Difco) by incubating for 3 h at 20°C before incubation for 45 h at 35°C. Tubes which showed growth with or without gas were streaked onto lactose agar and EMB agar and were transferred to EC broth and incubated at 44.5°C for 48 h. EC tubes with gas or growth were streaked onto lactose and EMB agar. Colonies were confirmed on the API 20E system.

Direct counts. Cells were enumerated by the acridine orange direct counting procedure of Hobbie et al. (12). A Zeiss standard 18 microscope was used with a 450- to 490-nm band pass filter, FT 510 beam splitter, LP 520 barrier filter, and a 100-W halogen lamp. Several modifications were made in the acridine orange direct counting procedure of Hobbie et al. (12). Nuclepore polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.) of 0.2-μm pore size were stained for 20 min in Irgalan Black (Ceiba-Geigy Corp., Greensboro, N.C.), and the filters, after three rinses in distilled water, were used damp. No surfactant was used (9). After staining with acridine orange, the filter was rinsed with 0.85% saline. The under-pad cellulose filter was not employed. The damp membrane filter was placed on the slide, a cover slip was placed directly on top of the filter, and the cells were counted immediately. A drop of nonfluorescent oil or saline was added to prevent rapid drying of the membrane.

Statistical analysis. By using a standard statistical program (SAS), analysis of variance was conducted. Calculated f values were compared with tabulated f values (17). Differences in the data were considered statistically significant at the 95% level of confidence.

RESULTS

Survival of V. cholerae serotype O1. The environmental V. cholerae serotype O1 strain (WF/1) survived well in estuarine waters and sediments. Figure 1 illustrates the effect of the inoculum size on the survival of the organism in estuarine waters and sediments. The initial inoculum had little effect on the number of organisms recovered from sterile sediments after 3 days. Although the number of viable cells recovered was initially greater in the estuarine water and sediment containing the highest inoculum, the differences were not significant. After 7 days, the organism grew abundantly in the sterile sediment and (data not shown) could be recovered after 35 and 60 days. At 35 days, the concentration of viable cells had declined to 10^4 cells per ml, and by 60 days the concentration had further decreased to 10^2 cells per ml. In nonsterile sediment, viable cells declined relatively quickly and were no longer recoverable after 12 days. Viable cells also decreased in nonsterile waters, although recovery was possible for up to 35 days.

The survival of WF/1 was examined in sediments (and waters) collected from two different sites in Apalachicola Bay during July. Site 36 was the location from which the organism was originally isolated, and site 29 was an area with a lower mean salinity located near the mouth of

![Fig. 1. Effect of inoculum size on the survival of V. cholerae O1 (WF/1) in estuarine waters and sediments incubated at 20°C. The values are expressed as colony-forming units.](http://aem.asm.org/Downloadedfrom/Vol.43,1982)
the Apalachicola River. Although the salinities of the waters were 6 and 15/100, there was no significant difference in the survival of the organism in the two different sediments (Fig. 2).

The survival of WF/1 varied with the time of year that water and sediment were collected (Fig. 3). Although there was no significant difference in the survival of the organism in sterile sediments collected during the three months, early recovery (less than 5 days) of viable cells in sterile water, nonsterile water, and nonsterile sediment was greatest in the September samples. However, recovery in nonsterile water and nonsterile sediment from any sample after 5 days was not significantly different.

The temperature at which the survival chambers were incubated had a significant effect on the survival of the organism (Fig. 4). The organism was observed to grow best at 35°C in sterile estuarine water and sediment. However, in nonsterile sediment at this temperature, the organism did not grow, nor could it be recovered after 5 days. At incubation temperatures of 25°C, recovery was limited to 5 days in nonsterile sediment and 10 days in nonsterile estuarine water. At 4°C, the organism did not grow in sterile sediments or waters, nor was it recovered after 5 days, but it survived for up to 15 days in nonsterile sediments and 10 days in nonsterile estuarine water.

Survival of other V. cholerae strains. Comparisons of different V. cholerae strains revealed no significant differences in the survival of serotype O1 and non-O1 isolates in sterile or nonsterile sediments or waters. No significant differences were noted in viable counts among V. cholerae O1 (WF/1) and non-O1 (WF/23 or WF/54) strains in sediments or waters (Fig. 5). There was also no significant difference in the survival of WF/1 and WF/103 as measured by viable counts (Fig. 6). Similarly, the clinical V. cholerae O1 isolate survived in sediments and water much like the environmental O1 isolate (Fig. 7). Direct counts of O1 and non-O1 strains showed no significant differences. Strains WF/1 and WF/108 (Fig. 8) and WF/1 and WF/111 (Fig. 9) exhibited the same response in sterile estuarine waters.

Survival of E. coli. In contrast to the V. cholerae strains, E. coli ATCC 25922 did not survive well in estuarine waters (Fig. 6) and could not be recovered by plating or most probable number procedures after 7 days in nonsterile water and 14 days in sterile water. In sterile sediment, E. coli did not survive as well as V. cholerae; but in nonsterile sediment, it actually survived better than the V. cholerae strains.

When a freshly isolated human strain of E. coli was examined, it also did not survive as well as the V. cholerae strains in sterile estuarine water (Fig. 9). Although E. coli most probable numbers were statistically the same as the viable counts, the direct counts were much higher. All attempts to resuscitate the intact E. coli cells by using lactose enrichment failed.

Direct and viable counts. The differences between direct counts and viable counts of V. cholerae strains in sterile estuarine water were far less than with E. coli strains. Viable counts
V. CHOLERAE AND E. COLI IN ESTUARIES

FIG. 3. Survival of V. cholerae O1 (WF/1) in estuarine waters and sediments collected during 3 different months and incubated at 20°C. The values are expressed as colony-forming units. Symbols: ■, sterile sediment; ●, sterile estuarine water; □, nonsterile sediment; ○, nonsterile estuarine water.

FIG. 4. Effect of temperature on the survival of V. cholerae O1 (WF/1) in estuarine waters and sediments. The values are expressed as colony-forming units. Symbols: ■, sterile sediment; ●, sterile estuarine water; □, nonsterile sediment; ○, nonsterile estuarine water.

of V. cholerae strains were 1 to 0.5 log less than direct counts, and the difference remained constant over the experimental period (Fig. 8 and 9). However, viable counts of E. coli were as much as 3 logs lower than direct counts; over the experimental period, both declined at different rates (Fig. 9).

The greatest difference between viable and direct counts of V. cholerae was observed with the adapted O1 strain (Fig. 9). Viable counts of this organism in sterile estuarine water were 2 logs lower than direct counts at day 0. However, after 2 days, the difference in direct and viable counts was only 0.5 log. Why this response occurs is uncertain, but the initial loss of viability of such a large population of cells may be a reflection of stress factors. These adapted O1 cells were nutrient starved, revitalized on Trypticase soy broth, placed in estuarine water, and then plated onto brain heart infusion agar. Exposure to such extreme conditions may result in an initial loss of viability on brain heart infusion agar.

DISCUSSION

Strains of V. cholerae, including environmental, clinical, and serotype O1 and non-O1 strains, showed no differences in survival rates in estuarine waters and sediments. The observed growth of V. cholerae in sterile sediment was probably due to the absence of competing organisms and the abundance of available nutrients. Gerba and McLeod (10) have demonstrated that autoclaving results in the release of bound nutrients from sediments. These nutrients would not be available in the natural environment. Therefore, the observed growth of V. cholerae in sterile sediment would be due, in part, to the availability of sediment-bound nutrients.
Growth and long survival were also noted in sterile estuarine waters and are probably due to the absence of competing organisms. In nonsterile waters, the organisms exhibited growth and survived for long periods of time, but they did not survive especially well in sediments. These results suggest that serotype O1 and non-O1 organisms are both well adapted to and capable of living in estuarine waters. Furthermore, the clinical isolate, exhibiting the same survival response, also appears to be well adapted to estuarine waters.

In contrast to *V. cholerae*, strains of *E. coli* did not survive well in estuarine waters. The fact that *E. coli* does not survive well in seawater alone but is able to survive better in the presence of marine sediments has been previously demonstrated (10). Our results confirm that a similar response occurs in estuarine waters and sediments. *E. coli* was recoverable from estuarine sediments for considerably longer than from waters. Furthermore, it survived even better in nonsterile sediments than *V. cholerae*.

The finding that *E. coli* does not survive as well as *V. cholerae* in estuarine waters has significant implications in terms of water and shellfish safety. Since fecal coliform *E. coli* levels are used to indicate the quality of water and shellfish, the question arises as to the validity of fecal coliforms as indicators of *V. cholerae*. One of the basic criteria for a good indicator organism is that the indicator must survive as long as the pathogen. The results of this study demonstrate that *E. coli* does not survive in...
estuarine water as well as *V. cholerae*. This suggests that measuring the levels of fecal coliforms in the water column may not be adequate for monitoring the presence of *V. cholerae*. Furthermore, there is increasing evidence from environmental studies that fecal coliform levels in estuarine waters do not correlate well with *V. cholerae* levels (8, 13, 14). Thus, if fecal coliform levels in estuarine waters do not indicate the presence of *V. cholerae*, other means of determining potentially dangerous levels of *V. cholerae* in estuarine waters and shellfish must be found.

**ACKNOWLEDGMENTS**

We gratefully acknowledge Ron Baker, John Cheney, and Kathy Williams for their excellent technical support, the
Florida State Department of Natural Resources for their logistical support, and R. R. Colwell for helpful discussions. The work is a result of research sponsored by Florida Sea Grant, National Oceanic and Atmospheric Administration, Office of Sea Grant, Department of Commerce, under grant SG # NA 80AA-D-00038.

LITERATURE CITED